# Methods for the Isolation and Characterization of Constituents of Natural Products

## XV. Application of a Periodic Acid Column for Locating Double Bond Position

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Common methods for locating double bond position are essentially variations of four reactions—ozonolysis (*2, 4, 6*), oxidation with osmium tetroxide (*9, 10*), oxidation with peroxy acids (*8, 17*), and oxidation with potassium permanganate in the presence of periodic acid or its salts (*1, 5, 7, 11*). Apparently little attention has been paid to the report that periodic acid itself can be used to locate double bonds (*3*). The lack of interest in this reaction may stem from the authors' statement that although the cleavage of the double bond with periodic acid proceeds smoothly with water-soluble substances, some difficulty might be encountered with water-insoluble substrates. We noted, however, while studying the regeneration of some 2,4-dinitrophenylhydrazone derivatives of unsaturated carbonyl compounds with periodic acid impregnated on magnesium sulfate, that some oxidation of double bonds occurred (*13*). The aldehydes resulting from the oxidation could be readily identified by converting them to 2,4-dinitrophenylhydrazones and subjecting these to thin-layer chromatography. Subsequently it was learned that ethylenic unsaturation in a number of classes of water-insoluble compounds could be satisfactorily located by this technique.

This report describes the method as applied to microgram quantities of fatty acids, their methyl esters, alcohols, and carbonyl compounds. In addition, the location of the double bond in the parent compound of the 2,4-dinitrophenylhydrazone derivatives of unsaturated aldehydes and in the 2,6-dinitrophenylhydrazone derivatives of the pyruvic acid esters of unsaturated alcohols has been accomplished and is also described.

## APPARATUS AND MATERIALS [1]

Dowex 50W × 8, anhydrous magnesium sulfate and $CCl_4$ were supplied by J. T. Baker Chemical Co., Phillipsburg, NJ. The $CCl_4$ gave a negligible blank and was used as received. Paraperiodic acid was obtained from the G. Frederick Smith Co., Columbus, Oh; disposable Pasteur pipets (145 × 7 mm o.d.) served as inexpensive columns. They were cut just below the crimp to facilitate insertion of column materials and were plugged with a small wad of glass wool.

## EXPERIMENTAL

The method is comprised of four steps: (1) oxidation of the ethylenic bond on passage of a $CCl_4$ solution of the compound through a column of $MgSO_4$ impregnated with periodic acid; (2) formation of 2,4-dinitrophenylhydrazone derivatives of the carbonyl fragments as they emerge from the periodic acid column, (3) removal of excess 2,4-dinitrophenylhydrazine on an ion-exchange resin; and (4) thin-layer chromatography of the derivatives. The entire procedure takes approximately 2 hr.

### *Preparation of Periodic Acid Column*

A volume of 1 ml of saturated aqueous periodic acid is ground with 5 g of $MgSO_4$ in a mortar until the mixture appears homogeneous. This is achieved by scraping the mortar several times with a spatula during the grinding process. The powder is sieved (80-mesh screen) and the material passing is stored at −18°C. If the powder is returned to the freezer immediately after each use, it is stable for up to one year.

A column is prepared by transferring 0.5 g of powder to a Pasteur pipet. It is packed by tapping on a bench top and then completely wetted with $CCl_4$. Air bubbles are removed by stirring with a wire. When the powder has resettled to a uniform packing, the flow rate should be slower than 20 min/ml of $CCl_4$. Approximately 30 min/ml is desired and this can be achieved by slight tamping.

### *Preparation of 2,4-Dinitrophenylhydrazine Column*

Celite impregnated with a phosphoric acid solution of 2,4-dinitrophenylhydrazine is prepared (*14*) and stored at −18°C. About 0.4 g of the mixture is packed under moderate pressure in a Pasteur pipet. Prior to use, 2-column volumes of benzene followed by 2-column volumes of $CCl_4$ are added to remove impurities.

*Preparation of Dowex-50 Column*

A Pasteur pipet is packed by pushing the end into the resin (used as received) so that a column approximately 30 mm in length will result when the resin is pushed lightly onto the glass wool plug. The resin is washed with 2-column volumes of $CCl_4$ prior to use.

*Column Setup for Reaction*

The columns are stacked from top to bottom as follows: periodic acid, 2,4-dinitrophenylhydrazine, and Dowex-50 with a 4-ml vial acting as receiver. The tips of the two top columns are shortened, if necessary, so that the tip just touches the top of the bed of the subsequent column.

*Procedure for Locating Double Bond*

The compound to be investigated (minimum 5 $\mu$g) is applied to the top column in 1 ml of $CCl_4$. When this has entered completely, the sides of the tube are washed with about 0.25 ml of $CCl_4$. This is followed by a column volume of $CCl_4$. The periodic acid column is removed and a column volume of $CCl_4$ is added to the next column and it is removed. The last column (Dowex-50) is then washed with a column volume of $CCl_4$.

*Identification of Oxidation Products*

The effluent from the column system (3–3.5 ml) is evaporated to dryness under a stream of $N_2$, the residue taken up in about 50 $\mu$l of benzene and an appropriate volume spotted on an alkaline thin-layer partition plate (*15*) along with authentic derivatives. The plate is developed with hexane. If, however, insufficient movement of the spots is observed, the plate may be developed with a more polar system such as hexane: benzene 63:35 (*15*).

Alkaline plates were used in this procedure because very small amounts ($< 0.5$ $\mu$g) of a hydrazone are readily visible. Neutral plates (*15*) may also be used and should be used if more polar hydrazones are expected. On neutral plates the spots may be intensified by spraying with methanolic KOH.

## RESULTS AND DISCUSSION

The compounds subjected to the oxidation, the expected fragments, and the fragments identified are listed in Tables 1 and 2. Reproductions of thin-layer chromatograms are shown in Figs. 1 and 2. In this study, 25 $\mu$g of the compound were passed over the columns, and one-fifth of the residue was analyzed by thin-layer chromatography. However, the double bond in several of the compounds was satisfactorily located

using the 5 $\mu$g for the oxidation and either all or part of the residue for thin-layer chromatography. On samples of 5 $\mu$g or less, a solvent blank should be run.

The colored substrates (Table 2, Fig. 2) as a rule showed more background than did colorless substrates (Table 1, Fig. 1), but a reliable determination of double bond position could still be made on 5 $\mu$g. The colorless substrates generally gave only the expected product, although some minor spots (about 2%) always were detected. In general, cleaner chromatograms were obtained from substrates containing the double bond located near the center of the chain.

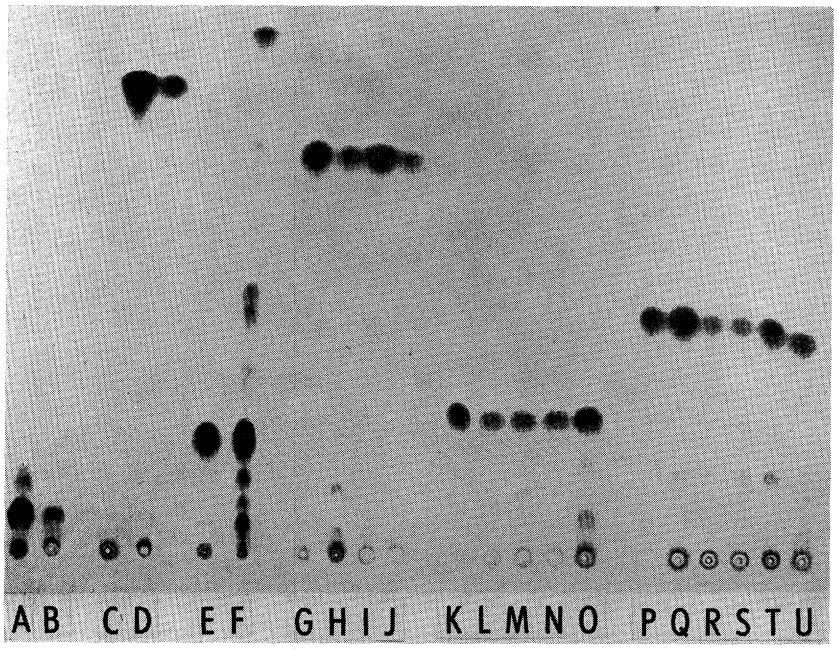


FIG. 1. Thin-layer partition chromatogram of unsaturated compounds oxidized on a periodic acid column. Spots are control 2,4-dinitrophenylhydrazones and 2,4-dinitrophenylhydrazone derivatives of carbonyl fragments produced by the oxidation. (A) propanal, (B) *cis*-3-hexen-1-ol products, (C) pentadecanal control, (D) methyl-*cis*-5-eicosenoate products, (E) hexanal control, (F) methyl-12-octadecenoate products, (G) dodecanal control, (H) methyl-*cis*-6-octadecenoate products, (I) petroselinic acid products, (J) methyl petroselinate products, (K) heptanal control, (L) vaccenyl alcohol products, (M) methyl-*trans*-vaccenate products, (N) methyl-*cis*-vaccenate products, (O) methyl palmitoleate products, (P) nonanal control, (Q) selachyl alcohol products, (R) methyl nervonate products, (S) elaidyl alcohol products, (T) oleyl alcohol products, (U) methyl oleate products.

Oxidation of compounds with terminal unsaturation either did not take place or else the formaldehyde escaped detection. Several of these were tried including methyl-10-undecenoate and 10-undecenoic acid. Citronellol and linalool, both of which would produce acetone, did not oxidize. No other compounds that would yield a ketone fragment were investigated.

A number of unsaturated 2,4-dinitrophenylhydrazones also failed to yield the expected fragment. Tiglaldehyde (2-methyl-2-butenal)-2,4-dinitrophenylhydrazone did not react to yield acetaldehyde. Since tiglaldehyde, citronellol and linalool all have a methyl group attached to

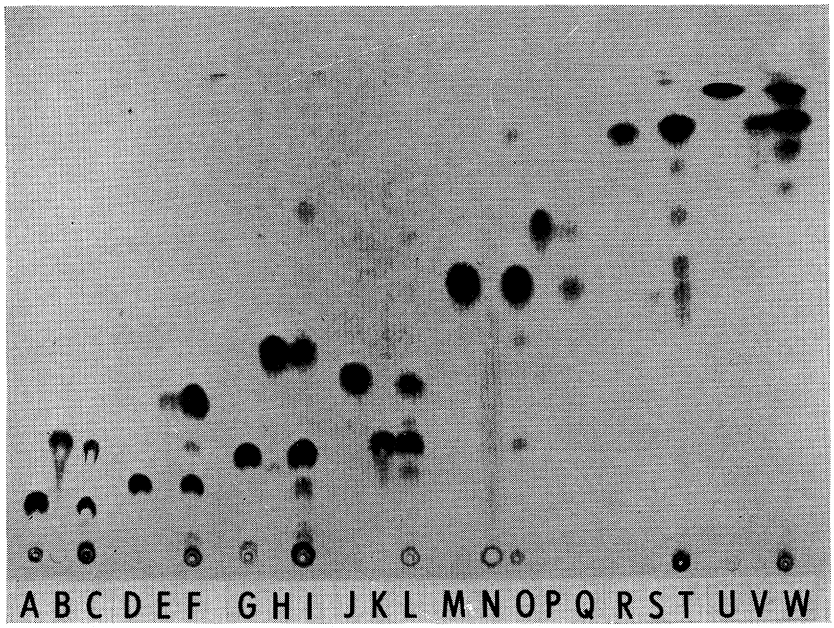


FIG. 2. Thin-layer chromatogram of unsaturated colored derivatives oxidized on a periodic acid column. Spots are control 2,4-dinitrophenylhydrazones and the 2,4-dinitrophenylhydrazone derivatives of carbonyl fragments produced in the oxidation. (A) propanal control, (B) 4-*cis*-heptenal-2,4-dinitrophenylhydrazone, (C) oxidized B, (D) butanal control, (E) 2-ethyl-hex-2-enal-2,4-dinitrophenylhydrazone, (F) oxidized E, (G) pentanal control, (H) 4-*trans*-nonenal-2,4-dinitrophenylhydrazone, (I) oxidized H, (J) heptanal control, (K) 2-nonenal-2,4-dinitrophenylhydrazone, (L) oxidized K, (M) nonanal control, (N) 9-octadecenyloxyacetaldehyde-$\alpha$-methyl-2,4,6-trinitrophenylhydrazone, (O) oxidized N, (P) oleyl alcohol ester of pyruvic acid 2,6-dinitrophenylhydrazone, (Q) oxidized P, (R) dodecanal control, (S) 2,4-hexadecadienal-2,4-dinitrophenylhydrazone, (T) oxidized S, (U) tetradecanal control, (V) hexadec-2-enal-2,4-dinitrophenylhydrazone, (W) oxidized V.

TABLE 1

COLORLESS COMPOUNDS SUBJECTED TO OXIDATION ON A COLUMN OF $MgSO_4$ IMPREGNATED WITH PERIODIC ACID

| Compounds | Source | Fragments expected | Identified |
|---|---|---|---|
| *Esters* | | | |
| Methyl nervonate | NCP[a] | Nonanal, methyl 14-formyl tetradecanoate | Nonanal |
| Methyl-*cis*-vaccenate | | Heptanal, methyl 10-formyl decanoate | Heptanal |
| Methyl-*trans*-vaccenate | | Heptanal, methyl 10-formyl decanoate | Heptanal |
| Methyl palmitoleate | | Heptanal, methyl 8-formyl octanoate | Heptanal |
| Methyl petroselinate | | Dodecanal, methyl 5-formyl pentanoate | Dodecanal |
| Methyl oleate | H[b] | Nonanal, methyl 8-formyl octanoate | Nonanal |
| Methyl-*cis*-5-eicosenoate | NMN[c] | Pentadecanal, methyl 4-formyl butyrate | Pentadecanal |
| Methyl-*cis*-6-octadecenoate | | Dodecanal, methyl 5-formyl pentanoate | Dodecanal |
| Methyl-12-octadecenoate | | Hexanal, methyl 11-formyl undecanoate | Hexanal |
| Methyl-10-undecenoate | A[d] | Formaldehyde, methyl 9-formyl decanoate | None |
| *Acids* | | | |
| Petroselenic | NCP | Dodecanal, 5-formyl pentanoic acid | Dodecanal |
| Oleic | H | Nonanal, 8-formyl octanoic acid | Nonanal |
| 10-Undecenoic | A | Formaldehyde, 9-formyl nonanoic acid | None |
| *Alcohols* | | | |
| Elaidyl | NCP | Nonanal, 9-hydroxy nonanal | Nonanal |
| Oleyl | H | Nonanal, 9-hydroxy nonanal | Nonanal |
| Vaccenyl | NCP | Heptanal, 11-hydroxy undecanal | Heptanal |
| Selachyl | F[e] | Nonanal, 9-octadecenyloxy acetaldehyde | Nonanal |
| Cinnamyl | MCB[f] | Benzaldehyde, glycolaldehyde | Benzaldehyde |
| Linalool | A | Acetone, formaldehyde, 4-ketopentanal | None |
| Citronellol | A | Acetone, 4-methyl-6-hydroxy hexanal | None |
| *Aldehydes* | | | |
| Crotonaldehyde | A | Acetaldehyde, glyoxal | Acetaldehyde, crotonaldehyde |
| Cinnamaldehyde | EK[g] | Benzaldehyde, glyoxal | Benzaldehyde, cinnamaldehyde |

[a] Nu Chek Prep, Elysian, MN.
[b] Hormel Institute, Austin, MN.
[c] Northern Marketing and Nutrition Laboratories, USDA, Peoria, IL.
[d] Aldrich Chem. Co., Milwaukee, WI.
[e] Fluka, Buchs, Switzerland.
[f] Matheson, Coleman & Bell, East Rutherford, NJ.
[g] Eastman Kodak, Rochester, NY.

TABLE 2

COLORED DERIVATIVES SUBJECTED TO OXIDATION ON A COLUMN OF $MgSO_4$ IMPREGNATED WITH PERIODIC ACID

| Compound | Fragments expected | Identified [b] |
|---|---|---|
| *Aldehydes* (as 2,4-dinitrophenylhydrazones) | | |
| Oleic | Nonanal, nonanedial | Nonanal [a] |
| 2-Ethyl-hex-2-enal | Butanal, $\alpha$-keto butanal | Butanal |
| Hexadec-2-enal | Tetradecanal, glyoxal | Tetradecanal |
| Hept-2-enal | Pentanal, glyoxal | Pentanal |
| *cis*-4-Heptenal | Propanal, butanedial | Propanal |
| *cis*-5-Heptenal | Acetaldehyde, pentanedial | Acetaldehyde [c] |
| Non-2-enal | Heptanal, glyoxal | Heptanal |
| *trans*-4-Nonenal | Pentanal, butanedial | Pentanal |
| *trans*-5-Nonenal | Butanal, pentanedial | Butanal |
| *trans*-6-Nonenal | Propanal, hexanedial | Propanal |
| *trans*-7-Nonenal | Acetaldehyde, heptanedial | Acetaldehyde [c] |
| *cis*-3-Hexenal | Propanal, propanedial | Not oxidized |
| Tiglaldehyde | Acetaldehyde, $\alpha$-keto propanal | Not oxidized |
| Hexadec-2,4-dienal | Dodecanal, but-2-en-dial, glyoxal | Dodecanal |
| *trans*-2-*trans*-6-nonadienal | Propanal, butanedial, glyoxal | Propanal |
| *Alcohols* (as esters of pyruvic acid 2,6-dinitrophenylhydrazone) | | |
| Oleyl | Nonanal, mixed derivative of 9-hydroxy nonanal | Nonanal |
| Cinnamyl | Benzaldehyde, mixed derivative of glycolaldehyde | Benzaldehyde |
| The $\alpha$-methyl-2,4,6-trinitrophenylhydrazone of octadec-9-enyloxyacetaldehyde | Nonanal, mixed derivative of 8-formyloctyloxyacetaldehyde | Nonanal |

[a] Some decanal also detected.

[b] Unoxidized colored substrates by virtue of their color are also seen on the plate.

[c] Several other unidentified spots on chromatogram.

one of the carbons of the double bond, it may indicate that this type of structure in general will not be oxidized on the periodic acid column.

The oxidation of the 2,4-dinitrophenylhydrazone of *cis*-3-hexenal proceeded quite well but the *trans* isomer did not react at all.

Yields of the carbonyl resulting from the oxidation were usually about 30–40% of theory. The yield was determined spectrophotometrically on the 2,4-dinitrophenylhydrazone of the aldehyde identified in the products of the oxidation from 25 $\mu$g of substrate. The yield, within limits, was dependent on the flow rate of the periodic acid column. A relatively rapid flow rate (20 min/ml) gave lower yields, and flow rates from 30–60 min/ml gave maximal yields. Flow rates slower than 60 min/ml did not increase the yield, but rather a small decrease was noted.

Besides $CCl_4$, other solvents including $CHCl_3$, $CH_2Cl_2$, benzene, cyclohexane and *n*-hexane were tried. All compounds underwent oxidation most readily in the nonpolar solvents. For example, selachyl alcohol (3-(9-octadecenyloxy)-1,2-propanediol) gave about 94% 9-octadecenyloxyacetaldehyde and 6% nonanal when $CH_2Cl_2$ was used as solvent. In $CCl_4$ no 9-octadecenyloxyacetaldehyde was observed, and the yield of nonanal increased to about 35% of theory. $CCl_4$ was the preferred solvent over the slightly less polar hexane because it gave a negligible blank without purification. Both hexane and cyclohexane were suitable solvents for carrying out the oxidation but gave high blanks despite being rendered carbonyl and alcohol free (*12, 14*).

Supports other than $MgSO_4$ were also tested. No oxidation took place on Celite 545, Analytical Grade Celite, Microcel T-38 or on glass micro beads. Celite and glass beads as a support for periodic acid oxidation of *vic*-glycols have also failed (16). Although $CaSO_4$ was suitable as a support to a certain extent, it proved to be inferior to $MgSO_4$.

Observations made during the course of this work indicated that under a given set of conditions, the ethylenic linkage in free fatty acids was more readily oxidized than in the other classes studied. The methyl esters appeared to be next, followd by the alcohols and carbonyls. This order of reactivity was determined by running the column at suboptimal speeds, that is, at a faster flow rate than 30 min/ml, and determining the yield of aldehyde produced from the oxidation of the double bond of the various classes studied.

It should be mentioned that a number of olefinic hydrocarbons including all possible unbranched monounsaturated decenes, and 9-octadecene were checked in both $CCl_4$ and hexane and found not to oxidize.

On the basis of the failure of Celite and glass as supports in the oxidation, the order of reactivity of the various classes studied, the observa-

tion that oxidation proceeds better in nonpolar solvents, and the inability of olefinic hydrocarbons to oxidize, the data suggests that adsorption may be a prerequisite for oxidation.

The procedure afforded the detection only of the carbonyl fragment produced on the hydrocarbon side of the double bond. The carbonyl fragment on the more polar side went undetected or unrecognized. The ester bond in the methyl esters of aldehydic acids produced from the oxidation of the fatty acid methyl esters are thought to be hydrolyzed by the periodic acid column (13). The 2,4-dinitrophenylhydrazones of other polar fragments such as aldehydic acids, hydroxyaldehydes and dialdehydes are either too insoluble in $CCl_4$ to be removed from the column or do not move in the thin-layer chromatography system. The method as described, therefore, is limited to the identification of only the carbonyl fragment from the hydrocarbon side of the double bond in monoethylenic compounds, and the hydrocarbon side of the ultimate double bond in polyunsaturated compounds.

Isomerism was observed in the 2,4-dinitrophenylhydrazones of most of the aldehydes produced from the oxidation when the hydrazones were spotted soon after they were formed. This was not a serious limitation in this study with pure or relatively pure starting compounds since the main isomer on the chromatogram always moved identically to the authentic expected fragment. It was noted, however, that if the hydrazones from the oxidation were stored dry for 48 hr or longer prior to chromatography, the faster-moving (weaker) isomer reverted quantitatively to the other form. This phenomenon should simplify interpretation of chromatograms that may be less readily interpretable, such as those obtained from impure starting material.

Although there are several limitations to the method, a number of advantages can also be claimed: (1) the procedure is relatively mild, (2) an ozonizer or gas chromatograph is not required; (3) short chain aldehydes, particularly acetaldehyde, if produced can be detected readily whereas this might be located under the solvent peak in gas chromatography, and (4) double bonds in colored derivatives can be located. To our knowledge this is the only method which can directly do this.

## SUMMARY

The ability of a microcolumn of periodic acid impregnated on magnesium sulfate to effect oxidation of ethylenic unsaturation was studied. The aldehyde, produced on the hydrocarbon side of the double bond in 30–40% yield, was converted to a 2,4-dinitrophenylhydrazone on a microderivatizing column and identified by thin-layer chromatography. The method was applied to unsaturation in fatty acids, their methyl esters, alcohols, aldehydes, and colored derivatives of the latter two classes. Double bonds in a variety of positions in the chain were

successfully located, but terminal unsaturation or unsaturation with a methyl group on either carbon of the double bond could not be characterized. The procedure was routinely run on 25 $\mu$g but can still be successfully executed on 5 $\mu$g of a pure compound.


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## REFERENCES


1. Bergelson, L. D., Dyatlovitskaya, E. V., and Voronkova, V. V., Complete structural analysis of fatty acid mixtures by thin-layer chromatography. *J. Chromatogr.* **15**, 191–199 (1964).
2. Beroza, M. and Bierl, Barbara, Rapid determination of olefin position in organic compounds in microgram range by ozonolysis and gas chromatography. *Anal. Chem.* **39**, 1131–1135 (1967).
3. Chatterjee, A. and Majumdar, S. G., Use of periodic acid for detecting and locating ethylenic unsaturation. *Anal. Chem.* **28**, 878–879 (1956).
4. Davison, V. L. and Dutton, H. J., Microreactor chromatography. Quantitative determination of double bond position by ozonization–pyrolysis. *Anal. Chem.* **38**, 1302–1305 (1966).
5. Downing, D. T. and Greene, R. S., Rapid determination of double bond positions in monoenoic fatty acids by periodate–permanganate oxidation. *Lipids* **3**, 95–100 (1968).
6. Edwards, H. M., Localization of unsaturation in fatty acids by identification of 2,4-dinitrophenylhydrazones derived from ozonides by thin-layer chromatography. *Lipids* **1**, 1–5 (1966).
7. Grimmer, G. and Jacob, J., Optimierung der Oxydativen Spaltung mit $MnO_4^-/IO_4^-$. I. Mitteilung:Monofettsäuren. *Z. Naturforsch.* **24b**, 565–568 (1969).
8. Kenner, G. W. and Stenhagen, E., Location of double bonds by mass spectrometry. *Acta Chem. Scand.* **18**, 1551–1552 (1964).
9. McCloskey, J. A. and McClelland, Martha J., Mass spectra of isopropylidine derivatives of unsaturated fatty esters. *J. Amer. Chem. Soc.* **87**, 5090–5093 (1965).
10. Perkins, E. G. and Argoudelis, C. J., Determination of double bond position in polyunsaturated fatty acids using combination gas chromatography–mass spectrometry. *Lipids* **4**, 619–621 (1969).
11. Rudloff, E. von, Periodate–permanganate oxidations. IV. Determination of the position of double bonds in unsaturated fatty acids and esters. *J. Amer. Oil Chem. Soc.* **33**, 126–218 (1956).
12. Schwartz, D. P., Quantitative microdetermination of alcohols as esters of pyruvic acid 2,6-dinitrophenylhydrazone. *Anal. Biochem.* **38**, 148–157 (1970).

13. Schwartz, D. P. and Brewington, D. R., Methods for the isolation and characterization of constituents of natural products. XIII. Regeneration of 2,4-dinitrophenylhydrazones on a periodic acid column. *Microchem. J.* **17**, (1), 63–67 (1972).
14. Schwartz, D. P. and Parks, O. W., Preparation of carbonyl-free solvents. *Anal. Chem.* **33**, 1396–1398 (1961).
15. Schwartz, D. P., Shamey, J., Brewington, C. R. and Parks, O. W., Methods for the isolation and characterization of constituents of natural products. X. New and improved methods for the analysis of carbonyl 2,4-dinitrophenylhydrazones and 2,4-dinitrophenylosazones. *Microchem. J.* **13**, 407–417 (1968).
16. Schwartz, D. P., Weihrauch, J. L. and Burgwald, L., Methods for the isolation and characterization of constituents of natural products. A periodic acid column procedure for the oxidation of *vic*-glycols, epoxides and $\alpha$-hydroxy acids at the micromole level. *Anal. Chem.* **41**, 984–986 (1969).
17. Vacherow, M. J., Michel, G. and Guilluy, R., Location of double bonds in unsaturated acids by mass spectrometry. *Bull. Soc. Chim. Biol.* **51**, 177–185 (1969).